Title

Malt protein inhibition of β-amylase alters starch molecular structure during barley mashing

Authors

Yu, Wenwen
Gilbert, Robert G
Fox, Glen P

Publication Date

2020-03-01

DOI

10.1016/j.foodhyd.2019.105423

Peer reviewed

Food Hydrocolloids 100 (2020) 105423

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: http://www.elsevier.com/locate/foodhyd

Malt protein inhibition of β-amylase alters starch molecular structure during barley mashing

Wenwen Yu [a], Robert G. Gilbert [b,c], Glen P. Fox [c,d,*]

[a] Department of Food Science & Engineering, Jinan University, Huangpu West Avenue 601, Guangzhou City, Guandong Province, 510632, China
[b] Joint International Research Laboratory of Agriculture and Agri-Product Safety of Ministry of Education of China, Yangzhou University, Yangzhou, 225009, Jiangsu Province, China
[c] The University of Queensland, Center for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, Brisbane, QLD, 4072, Australia
[d] Department of Food Science and Technology, 2158 Robert Mondavi Institute, University of California Davis, CA, 95616, USA

ARTICLE INFO

Keywords:
Barley
Protein
Starch
Mashing
Molecular structure
Amylase

ABSTRACT

The molecular structural changes in starch in barley malts during mashing (a major step in brewing beer) with and without protein removal were investigated using size exclusion chromatography. The aim was to uncover how proteins affects barley starch degradation in brewing. It was found that for malts containing lower β-amylase activity, protein removal significantly increased fermentable sugar content, whilst no significant change was observed for malts with higher β-amylase, with or without addition of a metalloprotease (Neutrase®). However, metalloprotease addition significantly reduced both the content and molecular sizes of remaining wort-soluble starches. This suggests that the effects of malt protein removal on starch degradation, particularly on fermentable sugar production, largely depend on malt enzyme activity, especially that of β-amylase. This provides useful information for brewers: for example, the fact that soluble starch molecular structure correlates significantly with fermentation efficiency gives a new criterion for selecting barley varieties for optimal brewing performance.

1. Introduction

Barley production yield ranks fourth among cereal crops following maize, rice and wheat (Asare et al., 2011). In total, around 90% barley is used for animal feed and for alcoholic beverage production. Malting, mashing and fermentation are the main processes involved in brewing (Yu, Quek, Li, Gilbert, & Fox, 2018). Malting is when barley grains are allowed to germinate for several days and then heated (up to 85 °C) to stabilize the enzymic activities (J. Wang, Zhang, Chen, & Wu, 2004). Mashing is when ground malted barley is mixed with hot water, leading to enzymatic hydrolysis of gelatinized carbohydrates (mainly starch) to fermentable sugars (Glen Fox, 2016). Many variables can influence mashing efficiency, e.g. mashing style (low-temperature ramping or high temperature infusion) (Glen Fox, 2016), malt starch molecular structures (Yu, Tao, Gidley, Fox, & Gilbert, 2019), malt amylolytic enzyme activities (Hu, Yu, et al., 2014), glucosidases (Serna-saldívar, Espinosa-Ramírez, & Esther, 2014), grist-to-water ratios, and levels of hydrolysis of starch and of protein during malting. While the fermentable sugars produced during mashing will be utilized by yeast in fermentation, numerous proteins from barley and synthesized during malting survive the mashing process (Schulz et al., 2018).

Starch is the most abundant component in barley, with a proportion between 62–77% (Asare et al., 2011). Thus, the hydrolysis of barley starch during mashing is a major factor in determining mashing performance, including the release of wort fermentable sugar content, and the amount of un-degraded wort soluble starches. Both of these significantly affect yeast fermentation (Serna-saldívar et al., 2014; Yu, Quek, et al., 2018). Many factors have been found that could significantly affect the hydrolysis of malt starch, thereby altering the production of both wort fermentable sugar and wort soluble starches (dextrins). For example, Slack, Baxter, and Wainwright (1979) found that malt protein (mainly hordein) can significantly inhibit starch degradation, as also seen in our previous study. In addition, enzyme activities, including those of α- and β-amylases and limit dextrinase, are also important in determining the production of fermentable sugars during mashing (MacGregor, Bazin, Macri, & Babb, 1999). It has also been found that malt starch molecular structure, including the chain length distribution and molecular sizes, also closely relate to mashing (Yu et al., 2019) and

* Corresponding author. The University of Queensland, Center for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, Brisbane, QLD, 4072, Australia.
E-mail address: g.fox1@uq.edu.au (G.P. Fox).

https://doi.org/10.1016/j.foodhyd.2019.105423
Received 14 July 2019; Received in revised form 22 September 2019; Accepted 4 October 2019
Available online 5 October 2019
0268-005X/

yeast fermentation efficiencies (Yu, Quek, et al., 2018). It was found in an in *vitro* system that barley protein could significantly inhibit starch hydrolysis at 37 °C (Yu, Zou, et al., 2018). Both total protein and total insoluble protein contents of malts have been found to significantly negatively correlate with wort fermentable sugar content (Yu et al., 2019). Consequently, it is reasonable to assume that malt protein has important effects on mashing performance (Schulz et al., 2018). No previous studies have demonstrated how malt protein inhibits starch hydrolysis during mashing or the possible underlying mechanism. Furthermore, little is known about protein effects on the properties of soluble starch remaining in wort (the liquid produced from mashing), which could significantly inhibit ethanol production during yeast fermentation (Yu, Quek, et al., 2018).

This study investigates how malted barley (malt) proteins affects starch degradation during mashing, including the release of fermentable sugar (glucose, maltose and maltotriose) as well as of wort soluble starches (dextrins). This study also investigates the effects of malt protein removal on starch molecular structural changes during mashing. Our hypothesis is that the removal of malt protein using Neutrase® (a metalloprotease) would significantly increase wort fermentable sugar content through promoting starch degradation instead of promoting the solubilization of starch during mashing. This is because at the mashing temperature (65 °C) and 1 h incubation, water molecules could still be absorbed by malt starch molecules, resulting in the commencement of starch gelatinization whether or not Neutrase was added. In addition, the addition of Neutrase would lead to a significant decrease of both content and the molecular size distributions of remained undegraded starches in the solid residue left after mashing (termed brewer's spent grain, BSG) and thus a more efficient mash extraction. The investigation of starch molecular structures of undegraded starches left in BSG may reveal the mechanism beneath the effects of malt β-amylase on starch degradation during mashing.

2. Materials and methods

2.1. Barley samples

Two malts with same genotype but grown in two locations in Australia were used, as described in our previous study (Yu et al., 2019) and shown in Table 1. Isoamylase (from Pseudomonas) and a total starch (AA/AMG) assay kit were bought from Deltagen Australia Pty. Ltd. (14 Pacific Place, Kilsyth Victoria, 3137, Australia). Pullulan standards with known peak molecular weights were from Polymer Standards (PSS) GmbH (Mainz, Germany). Dimethyl sulfoxide (DMSO, GR grade) was from Merck Co. Neutrase 0.8 L was from Novozymes Bagsvaerd, Denmark. All other chemicals were of reagent grade and used as received.

2.2. Micro-mashing

Mashing was conducted with 5 g grist (passed through a 500 μm sieve) with 20 mL distilled water kept in a 50 mL polyethylene flask and incubated in a water bath at 65 °C for 60 min, following the high-temperature infusion method of the Institute of Brewing (Fox, 2016). A stirrer bar was added into each flask and was set at 750 rpm for continuous stirring during the mashing process. Following this, the mixed mashing liquid was centrifuged at 4000×*g* for 10 min, after which the supernatant (termed wort) was poured into a 50 mL centrifuge tube, kept in boiling water for another 60 min to deactivate starch hydrolyzing enzymes, and then stored at −20 °C. The residue, BSG, was collected and freeze-dried for further analysis. Duplicate measurements were conducted for all malt samples.

2.3. Hydrolysis of malt protein with proteolytic enzyme during mashing

Neutrase® 0.8 L is a commercial enzyme used in the brewing industry (Hu, Dong, et al., 2014; Lund, Petersen, Andersen, & Lunde, 2015). It is a neutral, zinc metallo-endo-protease from *Bacillus amyloliquefaciens* that randomly hydrolyzes internal peptide bonds. In our experiments, before mashing, 300 μL Neutrase was added into each flask of the experimental group at the beginning of mashing, while for the control group, 300 μL distilled water was added instead.

2.4. Enzyme activities in barley malts

Enzyme activities of α- and β-amylase of malts were measured using the Malt Amylase Assay Kit (Megazyme International Ireland, Ltd). Limit dextrinase was measured using the Pullulanase/Limit Dextrinase Assay Kit (PullG6 Method; Megazyme). Biological and technical duplicate measurements were performed.

2.5. Enzyme activities in the mashing liquid at different mashing times

At the beginning and the end of mashing (10 and 60 min), 1 mL of the mashing liquid was collected and centrifuged at 6000×*g* for 10 min, with the residue being discarded. The supernatant was used for enzyme activity analysis as described above.

2.6. Sugars analysis of wort samples

Wort samples were diluted 20 times prior to analysis of the fermentable sugar profile using a method described previously (Yu, Quek, et al., 2018; Yu et al., 2019). Standard mixtures of glucose, maltose and maltotriose were made in the range 1–27 μg/mL in water. Samples were analyzed on an Agilent 1100 HPLC with ELSD detection. The HPLC solvent was 75% acetonitrile in water, with a 1 mL/min flow rate using an Alltech Carbohydrate column, 4.6 mm × 250 mm. The ELSD was set to a nitrogen flow rate of 2 mL/min (at 87 °C). Duplicate measurements were performed for each wort.

Table 1
Chemical compositions and thermal properties of barley malt flour[a].

Chemical composition of barley malts[1]						
Sample ID	Genotype	Location	Protein content/%[2]	Moisture content/%	Starch content/%[2]	Amylose content/%
82M	Gairdner	Charlick	$(6.6 \pm 0.2)^a$	$(2.6 \pm 0.3)^a$	$(41.7 \pm 0.4)^a$	$(32.1 \pm 0.3)^b$
89M	Gairdner	Roseworthy	$(11.8 \pm 0.1)^b$	$(3.6 \pm 0.8)^b$	$(44.3 \pm 0.4)^b$	$(31.6 \pm 0.1)^a$
Gelatinization properties[1]						
	T_o/°C	T_p/°C	T_c/°C	Enthalpy/J g–1		
82M	$(55.2 \pm 0.2)^a$	$(60.5 \pm 0.0)^a$	$(64.8 \pm 0.0)^a$	$(4.5 \pm 0.3)^b$		
89M	$(56.2 \pm 0.1)^b$	$(62.6 \pm 0.1)^b$	$(67.9 \pm 0.6)^b$	$(2.9 \pm 0.1)^a$		

[1] All data was based on duplicate measurements and repeated from Yu et al. (2019).
[2] Data was on dry basis. Samples with different letters in the same column are significantly different at $p < 0.05$.

2.7. Soluble starch content in wort

Soluble starch content in wort was measured using the method given in the Megazyme Total Starch kit (K-TSTA-1107) bought from Deltagen Australia Pty. Ltd. (14 Pacific Place, Kilsyth Victoria, 3137, Australia).

2.8. BSG chemical composition

The moisture content of BSG was measured by drying samples in a vacuum oven at 110 °C overnight and recording the weight loss in triplicate. Starch content in BSG was measured using the Total Starch kit. Before measuring the starch content, the weighed BSG flour was washed using 2 mL absolute ethanol and centrifuged at 4000×*g* for 10 min. The residue was then washed again with 2 mL absolute ethanol and centrifuged again. The residue was used for the measurement of total starch content. The crude total protein content of BSG was calculated from the nitrogen content determined using a Leco CNS-2000 analyzer on carbon, nitrogen and sulfur (Seminole, Florida, USA) with a conversion factor of 6.25; both biological and technical duplicate measurements were conducted.

2.9. Starch extraction

Starch extraction from malts and BSG was conducted using a method described previously (Yu et al., 2019). In summary, after removing protein by protease and centrifuging at 4000×*g* for 10 min, 0.5 mL of the lichenase solution (100 μL enzyme mixed with 2 mL sodium phosphate buffer (20 mM, pH = 6.5; Megazyme)) was added and was kept at 40 °C for 1 h to remove β-glucan. After centrifuging (4000×*g*, 10 min), the residue was re-dissolved overnight in 1.5 mL dimethyl sulfoxide (DMSO, Sigma-Aldrich) containing 0.5% LiBr at 80 °C for SEC analysis.

Soluble starch in wort was extracted using the method 'Determination of starch in samples in which the starch is present in a soluble form and D-glucose and maltodextrins are present' from the Megazyme Total Starch kit. In brief, 2 mL worts were mixed with 8 mL 95% ethanol to precipitate wort-soluble starch over 30 min and then centrifuged (6000×*g*, 10 min), with the supernatant discarded. The residue was redissolved with 1 mL warm distilled water before mixing with 0.5 mL protease in tricine buffer (pH = 7.5, 250 mM, adjusted with 0.1 M NaOH) at 37 °C for 30 min. After this, another 8 mL of 95% ethanol was added into the solution to precipitate soluble starch for 30 min. After centrifuging (6000×*g*, 10 min), with the supernatant discarded, the residue was again steeped with 0.5 mL of 0.45% (w/v) sodium bisulfite solution for another 30 min. The residue containing soluble starch was mixed with 8 mL 95% ethanol to re-precipitate soluble starch. After centrifuging (4000×*g*, 10 min), the residue containing starch was further mixed with 1 mL lichenase solution (100 μL enzyme mixed with 2 mL sodium phosphate buffer (20 mM, pH = 6.5, Megazyme)) and kept at 40 °C for an hour to remove remaining β-glucan. After removing the β-glucan, the mixture was mixed with 8 mL 95% ethanol to re-precipitate starch. After centrifuging (4000×*g*, 10 min), the residue was re-dissolved overnight in 1.5 mL DMSO/LiBr at 80 °C.

Starch that re-dissolved in DMSO/LiBr was used directly for branched starch structural analysis.

2.10. Starch debranching

For debranched analysis, starch dissolved in DMSO/LiBr overnight at 80 °C was further precipitated using 6 mL absolute ethanol (twice). After centrifuging (4000×*g*, 10 min), the residue was mixed with 0.9 mL hot distilled water and kept in boiling water for at least 15 min until the starch was completely gelatinized. After cooling to room temperature, starch was debranched using isoamylase following a method described elsewhere (K. Wang, Hasjim, Wu, Henry, & Gilbert, 2014).

2.11. Size-exclusion chromatography (SEC)

Extracted starches, both branched and debranched, dissolved in DMSO/LiBr were analyzed using a Waters SEC-MALLS system (Wyatt Technology), equipped with dual detectors: differential refractive index (DRI) and multiple-angle laser light scattering (MALLS) using the method described elsewhere (Tao, Li, Yu, Gilbert, & Li, 2019; Yu et al., 2019). SEC separates by molecular size, specifically the hydrodynamic radius R_h, not by molecular weight. For complex branched whole starch molecules, there is no relation between molecular size and molecular weight. However, for linear polymers, like debranched starches, there is a unique relationship between size and molecular weight, for which one can find the weight CLD as a function of the degree of polymerization (DP) (X), $w(\log X)$. The relation between the number CLD (the number of chains of a given degree of polymerization (X) following debranching, $N_{de}(X)$), and the corresponding weight distribution is $w(\log X) = X^2 N_{de}(X)$ (Castro, Ward, Gilbert, & Fitzgerald, 2005). The relationship between the SEC elution time, R_h and X for linear polymers is found by calibration with known standards and the Mark- Houwink equation (Vilaplana & Gilbert, 2010). It is important to be aware that the R_h and X values resulting from this involve a number of assumptions, and while trends are certainly correct, the actual values are subject to some uncertainty.

2.12. Statistical analysis

Two-tail tests were carried out to determine significant differences between two different factors; $p \leq 0.05$ and $p \leq 0.01$ were used as thresholds of significance and high significance, respectively. Statistical significance was analyzed using one-way ANOVA with Duncan adjustment at $p < 0.05$, with IBM SPSS Statistics version 21.

3. Results and discussion

3.1. Chemical composition of barley malt

The two malts used in this study were the same as used in our previous study (Yu et al., 2019), but for convenience, the chemical composition and gelatinization characteristics of malts are repeated in Table 1. For both malts, the peak gelatinization temperatures are below the standard mashing temperature of 65 °C that we used in this study. Thus, there should be no limitation on the rates of starch solubilization and enzyme-substrate interaction.

3.2. Content of fermentable sugars in wort

Fig. 1 shows the wort fermentable sugars profile including glucose, maltose and maltotriose. Maltose was the most abundant sugar, followed by maltotriose and then glucose. For mashing without Neutrase addition, sample 89M released a slight but significantly higher amount of maltose than 82M (Fig. 1). This is probably because of the higher activity of amylolytic enzymes, particularly β-amylase in 89M (Fig. 5c), as reported previously (Yu et al., 2019). On the other hand, following Neutrase addition, it was found that the content of maltose released by 82M during mashing was significantly higher than that released from 89M. For 82M, the addition of Neutrase significantly increased the wort fermentable sugar content, particularly of maltose and maltotriose, whilst for 89M, the addition of Neutrase did not significantly change wort fermentable sugars.

3.3. Chemical compositions of BSG and wort soluble starch content

Table 2 shows that Neutrase addition significantly decreased the total protein content in BSG, which in turn was probably because of the increased BSG starch content. Although Neutrase is used to remove malt protein during mashing, there was about 10% of total protein remaining

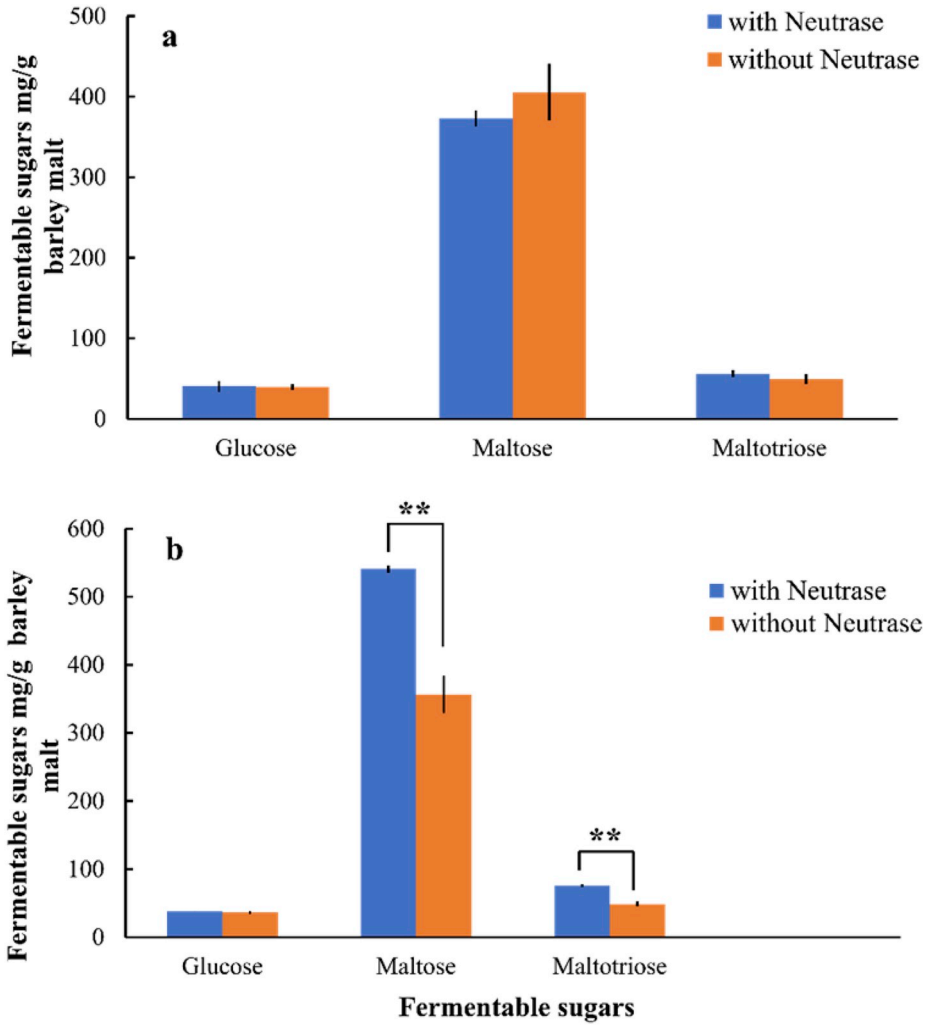

Fig. 1. Fermentable sugar contents in wort with and without the addition of Neutrase during mashing. a, sample 89M; b, sample 82M. Error bars are standard deviations; **, data significantly different at $p < 0.01$. Data from biological and technical duplicates.

Table 2
Starch content in BSG and in wort after mashing[1].

	Mashing	BSG			Wort
	Starch solubilization/%	Moisture content/%	Starch content/%[2]	Protein content/%[2]	Soluble starch (mg/mL)
82M with Neutrase	$(90.9 \pm 3.1)^{a}$	$(6.3 \pm 2.2)^{b}$	$(27.1 \pm 2.6)^{c}$	$(9.4 \pm 0.1)^{d}$	$(3.7 \pm 0.1)^{b}$
82M without Neutrase	$(92.0 \pm 1.3)^{a}$	$(3.6 \pm 0.7)^{ab}$	$(19.4 \pm 0.3)^{ab}$	$(17.5 \pm 0.0)^{b}$	$(6.8 \pm 0.8)^{c}$
89M with Neutrase	$(91.0 \pm 0.3)^{a}$	$(3.0 \pm 0.3)^{a}$	$(23.9 \pm 3.4)^{bc}$	$(10.0 \pm 0.4)^{c}$	$(1.8 \pm 0.0)^{a}$
89M without Neutrase	$(90.6 \pm 0.1)^{a}$	$(2.1 \pm 0.1)^{a}$	$(16.6 \pm 0.6)^{a}$	$(27.9 \pm 0.4)^{a}$	$(5.4 \pm 0.6)^{c}$

[1] Data based on both biological and technical duplicate measurements.
[2] Data on dry basis.

in the samples. This incomplete hydrolysis has also been reported by Bi et al. (2018). It is possible that mashing at 65 °C may reduce the enzyme activity of Neutrase, which has an optimal temperature of 40–50 °C. Moreover, the hydrolysate (Kammoun, Bejar, & Ellouz, 2003) and the enzyme activity of amylase (Pliansrithong, Usansa, & Wanapu, 2013) can reduce the enzyme activity of Neutrase. Nevertheless, Neutrase hydrolyzes most malt proteins.

As clearly shown in Table 2, the addition of Neutrase did not significantly increase the solubilization of malt starch during mashing. This indicates that during mashing, malt protein is not the reason inhibiting starch solubilization. This is also in accordance with a previous finding, where no significant correlation was found between malt protein content and the amount of starch solubilized during mashing (Yu et al., 2019).

Comparison of the content of soluble starches remaining in the wort showed that for both malts, the content of soluble starches in wort with protein removal was significantly lower than that of wort produced from malts without Neutrase addition. This suggests that although during mashing, the addition of Neutrase did not significantly alter the percentage of starch that solubilized into the mashing liquid, it significantly reduced the content of solubilized starches. Thus it is probable that during mashing, after the addition of Neutrase, more amylolytic enzyme was released into the solution (Hu, Dong, et al., 2014), and/or that the removal of malt protein by Neutrase also increased the enzyme activity through reducing protein binding interactions, thereby increasing soluble starch degradation, as reported for both barley (Yu, Zou, et al.,

2018) and wheat flour (W. Zou, Sissons, Gidley, Gilbert, & Warren, 2015).

3.4. Starch molecular structure in wort

Both the content and aspects of the molecular structure of wort-soluble starches have been reported to have significantly negative correlations with ethanol production (Yu, Quek, et al., 2018). We here examine the effects of malt protein on wort-soluble starch molecular structures.

It is possible that the peak existing around $R_h = 1$–3 nm seen in the SEC is remnant undegraded protein and/or smaller soluble polysaccharides, particularly for those of rice starch, as postulated elsewhere (Syahariza, Sar, Tizzotti, Hasjim, & Gilbert, 2013; Tao et al., 2019). However, this may not be the case for wort starches. As shown in Fig. 2, comparison of the SEC weight distributions of branched and debranched wort-soluble starches shows that whether or not Neutrase was added during mashing, after debranching, the amount of starch molecules with $R_h < 3.5$ nm decreased significantly. It was also observed that for starch molecules with $R_h \gtrsim 3.5$ nm, no significant change was seen before and after debranching. This in turn suggests that for those molecules with $R_h \lesssim 1.2$ nm after debranching (whether or not Neutrase has been added) must arise from the debranching by isoamylase of soluble starches with R_h between 1.2–3.5 nm. Consequently, the smaller molecules with R_h ranging from 1.2–3.5 nm, are probably starch rather than protein. This hypothesis is supported by the fact that during extraction, 95% ethanol was used to precipitate starches followed by protease addition. However, it cannot be unambiguously concluded here that there is no possibility at all that no protein molecules remained undegraded. On the other hand, as shown in Fig. 2, for both malts, no significant change for starch polymers with $R_h \gtrsim 3.5$ nm was observed before or after debranching. This suggests that wort-soluble starches with $R_h >$~3 nm are largely linear molecules.

Even though the above explanation does not prove unambiguously that there was no protein remaining in SEC signals with R_h 1.2–3.5 nm, the results still showed that there must be a large amount of fully branched starch molecules in worts with $R_h < 3.5$ nm which could be effectively enzymatically debranched using isoamylase (Fig. 2). Considering this, as shown in Fig. 2a and b, for both malts, Neutrase addition must have significantly decreased the content of wort-soluble starches with R_h ranging from 0.35 to ~35 nm, particularly for starch molecules with $R_h < 3.5$ nm. The comparison in the molecular weight distribution of soluble starches with same sizes with and without Neutrase addition indicates that protein removal has debranched starch molecules as well (Fig. 3c and d). It is thus probable that protein removal increased the enzyme activity of debranching enzyme, which in malt is limit dextrinase (Bøjstrup, Marri, Lok, & Hindsgaul, 2015).

In addition, as shown in Fig. 3 e & f, after debranching, it was found that removal of malt protein not only significantly decreased the amount of starch chains with DP ranging between 35–3500, the removal of malt protein with varied protein content also significantly decreased the amount of starch chains with DP < 35. The amount of very small molecules significantly decreased after the addition of Neutrase, suggesting that these contain proteins.

3.5. Starch molecular structure in BSG

To further investigate how malt protein removal during mashing affects starch degradation, the molecular structure of remaining undegraded starch after mashing with and without Neutrase addition was measured and is shown in Fig. 4 and Fig. S1. Similar to wort-soluble starches (Fig. 2), for both malts, after debranching there was a significantly decrease of the amount of starch molecules with $R_h \gtrsim 2.2$ nm, whether or not Neutrase was during mashing. This suggests again that although it is possible that for the smaller molecules in SEC could be protein residues, there was nevertheless a large amount of smaller starch granule also remaining in BSG.

As shown in Fig. 4, compared with the native malt starch, mashing

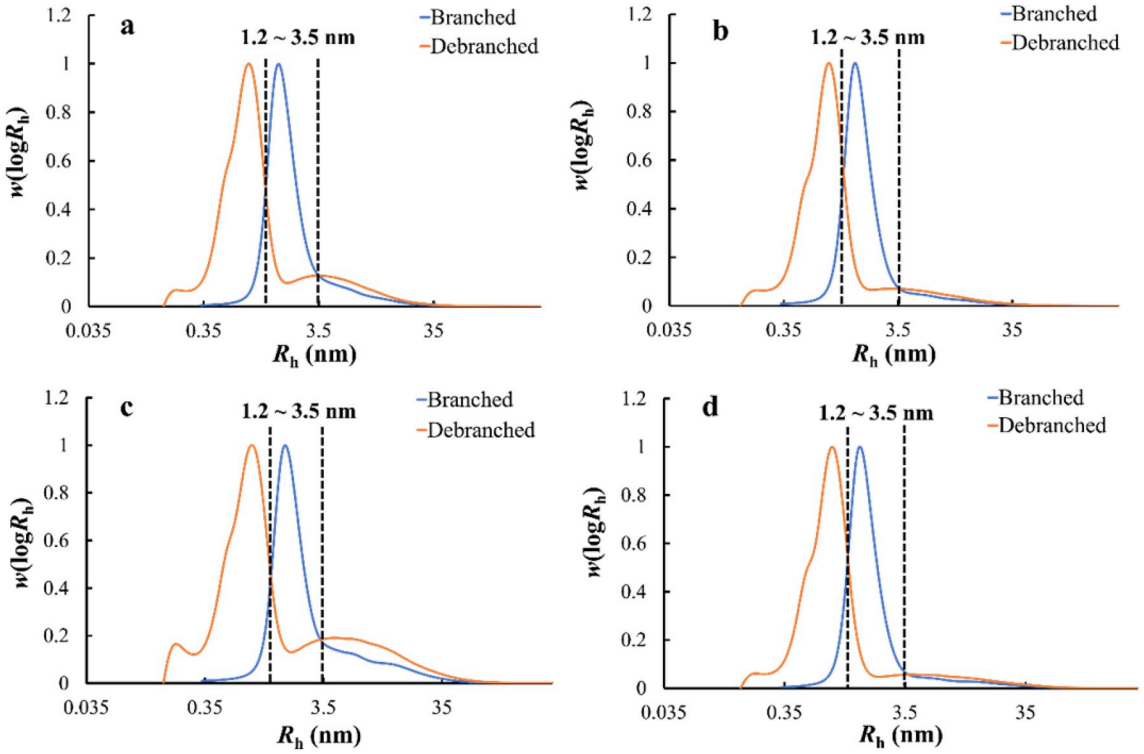

Fig. 2. Comparison of SEC weight distributions, $w(\log R_h)$, as a function of molecular sizes, R_h of fully-branched and isoamylase debranched wort-soluble starches of sample 82M (a & b) and 89M (c & d). a & c, with the addition of Neutrase and b & d without Neutrase addition during mashing. All data from both biological and technical duplicates.

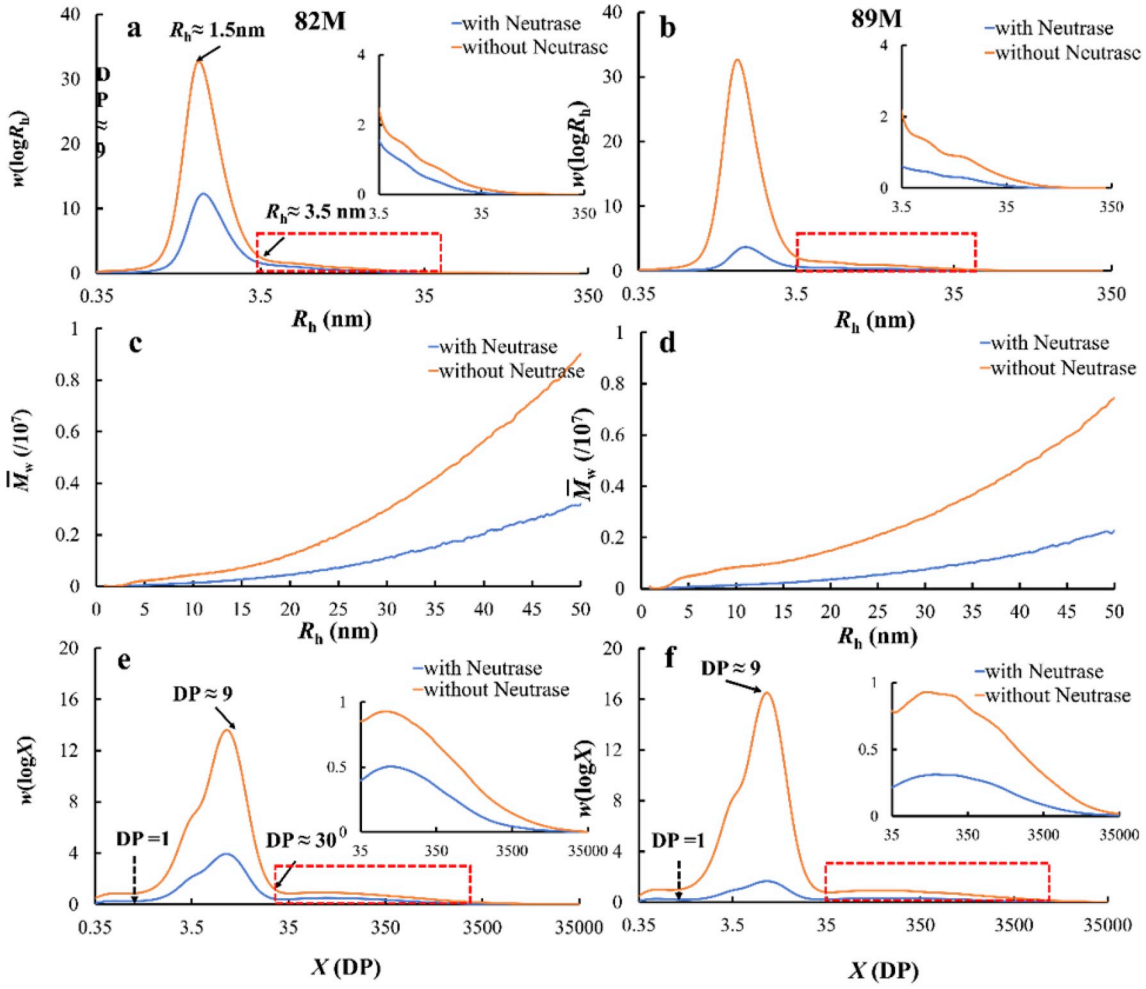

Fig. 3. The comparison of SEC weight distributions, $w(\log R_h)$, as a function of molecular sizes, R_h of fully-branched wort-soluble starches with and without Neutrase addition during mashing, for 82M (a) and 89M (b); the weight-average molecular weight $\overline{M}_W$ of whole wort soluble starches as a function of R_h (note the different scales on the Y axis) with and without Neutrase addition of 82M (c) and 89M (d); and the SEC weight CLDs, $w(\log X)$, of debranched wort soluble starches as a function of DP (X) with and without Neutrase addition of 82M (e) and 89M (f). All peaks were normalized to the total wort soluble starches for better comparison of the effects of Neutrase addition on starch molecular structural change during mashing. The corresponding enlarged part of the red frame of each graph is also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

without Neutrase addition, BSG from 82M contained almost no starch molecules with $R_h > 100$ nm, whilst BSG from 89M contained a significantly higher amount of starch molecules above this size. This suggests that malt protein largely inhibited the hydrolysis of larger starch molecules, particularly of those with $R_h > 10$ nm, as reported previously (Yu et al., 2019; Wei; Zou, Sissons, Warren, Gidley, & Gilbert, 2016).

Following the addition of Neutrase after mashing, it was found that there was a higher amount of larger starch molecules remaining undegraded for both malts, with $R_h > 7$ nm and $R_h > 15$ nm for 82M and 89M, respectively (Fig. 4 a & b), compared with that when no Neutrase was added. Furthermore, after debranching, it was found that compared with no Neutrase addition, more starch branches with DP $\gtrsim$ 100 and $\gtrsim$ 230 for 82M and 89M respectively remained undegraded after mashing (Fig. 4 c & d), when Neutrase was added during mashing.

It is possible that the CLD of debranched starches extracted from BSG may still contain peaks of residual protein molecules, but as shown in Fig. 4. C & d, after the addition of Neutrase, there were fewer starch chains with DP 8–100 remaining in the BSG, compared with that without Neutrase addition. This indicates that during mashing, while the removal of malt protein could significantly increase the hydrolysis of shorter starch branches, it also significantly reduced the hydrolysis of longer starch branches. This finding is in accordance with a previous study showing that the pre-treatment of maize starch with barley β-amylase could significantly inhibit the degradation of larger starch molecules while significantly reducing the content of short starch chains (Ao et al., 2007).

3.6. Enzyme activities of barley malts during mashing

To investigate the mechanism for the effect of malt protein on starch degradation during mashing, both α-, β-amylase and limit dextrinase of native malt and of the mashing liquid at different time points were analyzed. As shown in Fig. 5, for α- and β-amylases and limit dextrinase, enzyme activity during the first 10 min of mashing was significantly lower than that of the original malt enzyme. This is probably because not all enzymes were released into the mashing liquid during the first 10 min.

As shown in Fig. 4, during the mashing process, after limit dextrinase was formed, the system lost almost all enzyme activity after 10 min, and there was only a slight decrease of α-amylase enzyme activity during the whole mashing process. Even at the end of mashing, no significant change of α-amylase activity was observed, compared with that at 10 min of mashing. Compared with limit dextrinase, β-amylase gradually lost its activity during the whole mashing process (MacGregor et al., 1999). The varied thermal properties of these three amylolytic enzymes would have significant effects on starch degradation and also on wort

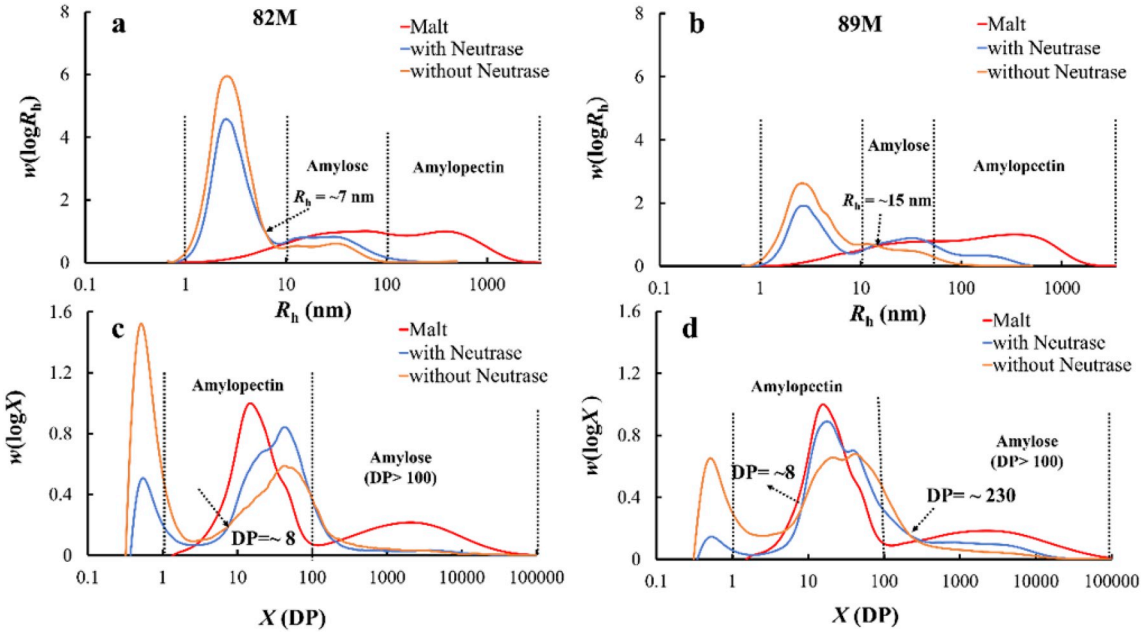

Fig. 4. SEC weight distributions of whole starches extracted from malts and BSG $w(\log R_h)$ as a function of molecular sizes R_h (a, 82M, b, 89M), and SEC weight CLDs, $w(\log X)$, of debranched starch extracted from malts and BSG (c, 82M, d, 89M). All peaks were normalized to the total weight of starches used for mashing. Data from biological and technical duplicates.

fermentable sugars production, as limit dextrinase hydrolyzes the 1 → 6 branches on amylopectin and amylose and thereby reduces the number of chains for the amylase to hydrolyze to shorter chains to form maltose.

It was found that after the addition of Neutrase, the enzyme activity of β-amylase of both malts was significantly higher than that without Neutrase addition. This is in accordance with previous findings reported by Hu, Dong, et al. (2014). However, it should be noted that Neutrase can also partially hydrolyze malt β -amylase, as reported by Bi et al. (2018) in a study where the temperature was set at 60 °C (very close to the mashing temperature used in this study). Our results showed that the enzyme activities of β -amylase released with the addition of Neutrase was significantly higher than that of the control group (malt, Fig. 5). Further investigation might identify how Neutrase on alters the activity of β-amylose and limit dextrinase. It is reasonable to assume that malt protein significantly affects starch degradation during mashing by inhibiting the release of enzymes, particularly of β-amylase.

4. Discussion

This study investigated how the addition of an exogenous proteolytic enzyme, Neutrase, on malt starch degradation during mashing, and considered the underlying mechanism. The results showed that Neutrase addition did not significantly improve the percentage of malt starch solubilized during mashing (Table 2). It is supposed that the presence of protein bodies and other substances surrounding the starch granules could decrease the water absorption/uptake into the granules, and thereby could decrease starch gelatinization (Yu et al., 2019), as has been found with sorghum flour (Chandrashekar & Kirleis, 1988). However, at the mashing temperature used here (65 °C), which was higher than that of the peak gelatinization temperature of 82M and 89M (Table 1), and after 1 h incubation, it is likely that water molecules could still penetrate into and be absorbed by malt starch molecules, resulting in partial malt-starch gelatinization, whether or not Neutrase was added. This hypothesis is supported by our previous finding that there was no significant correlation between malt protein content and the proportion of starch solubilized after mashing (Yu et al., 2019).

Our results showed that the addition of Neutrase significantly increased the activities of both β - amylase and limit dextrinase (Fig. 5). The hydrolysis of malt protein by Neutrase would have also resulted in more exposed starch granules. In this way, it is not hard to understand that significantly lower content of soluble starches, particularly for those with small molecular sizes and short chain lengths ($R_h < 3.5$ nm, DP < 30), was found in the wort in this study, which can have significant effects on properties (Gong, Cheng, Gilbert, & Li, 2019; Guo, 2018). Our results also showed that for both malts, after Neutrase addition, the remaining un-degraded starches in BSG had significantly different molecular structures than in those without Neutrase addition (Fig. 4). Particularly, the addition of Neutrase resulted in a significantly higher amount of starch molecules with larger sizes ($R_h > 7$ nm for 82M and $R_h > 15$ nm for 89M, Fig. 4a) remaining undegraded. Further, the DP of these larger starch molecules remaining in BSG after mashing was above >100 and above >230, respectively (Fig. 4 c & d). In addition, fewer starch branches with medium chain lengths (DP < 8 for 82M, DP < 8 and DP over the range 37–230 for 89M, respectively, Fig. 4 c & d) remained in the BSG, compared with that without Neutrase addition.

This finding is in accordance with a previous study where it was concluded that the pre-treatment of maize starch with barley β-amylase significantly increased the remaining undegraded larger starch molecules while significantly reducing the amount of smaller starch molecules with short chain lengths (Ao et al., 2007). It is reasonable to assume that the differences in the effects between samples of removal of protein is due to the difference in malt amylolytic enzymes, particularly the release of β -amylase after protein removal using Neutrase.

However, unlike the effects on malt starch structural change, here it was found that the addition of Neutrase led to very different results for wort-fermentable sugars in different malts, particularly with regard to maltose content (Fig. 1). For 82M, the increased enzyme activity resulting from Neutrase addition significantly increased the content of all fermentable sugars, whilst for 89M containing higher β-amylase activity, no significant change in wort fermentable sugar content was observed. Our hypothesis is that the effects of protein removal on wort fermentable sugar content largely depend on the original malt enzyme activity, particularly β-amylase and/or on the original malt protein content, as malt β-amylase enzyme activities usually significantly and positively correlate with total malt protein content (Yu et al., 2019). That is, for malt with less β-amylase, the removal of malt protein during

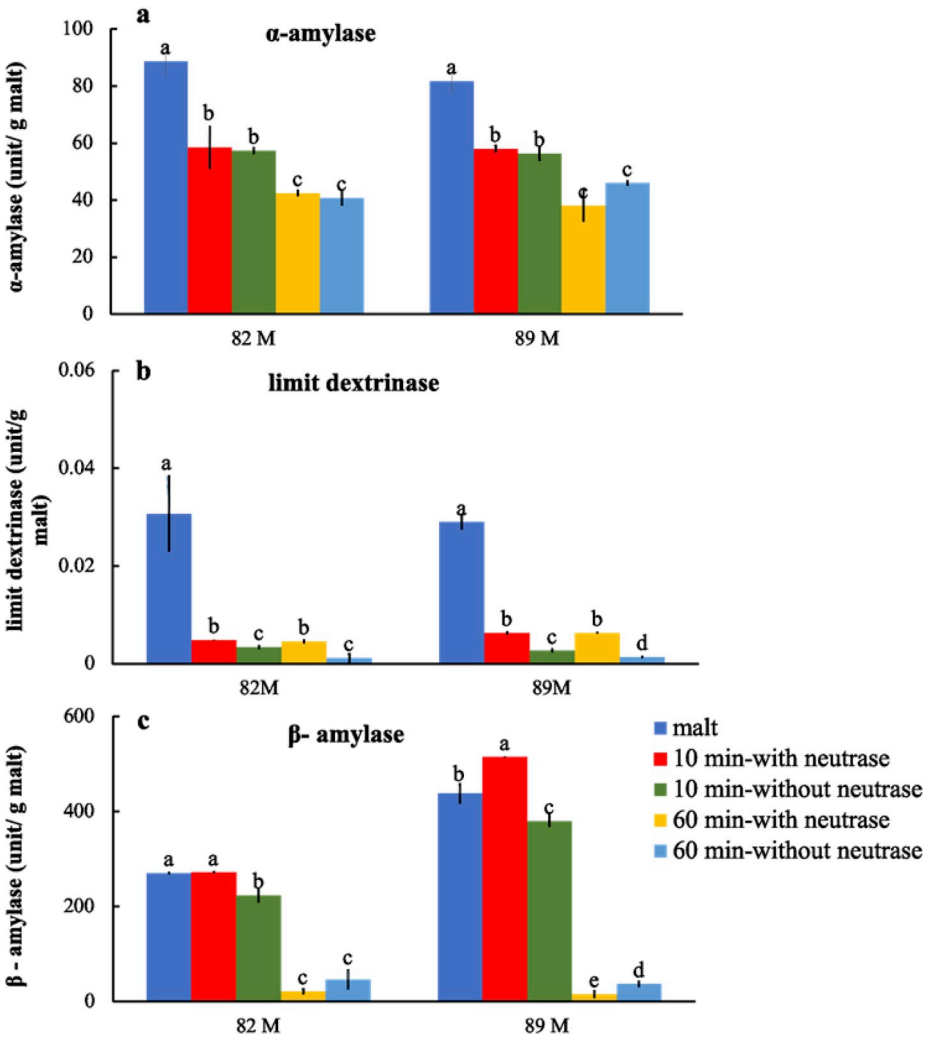

Fig. 5. Enzyme activities of α-amylase (a), limit dextrinase (b) and β-amylase (c) of barley malts and of the mashing liquid at 10 min and 60 min. For each graph, values with different letters represent significant different at $p < 0.05$. Data from biological and technical duplicates.

mashing could significantly increase wort fermentable sugar contents (mainly maltose and maltotriose, Fig. 1b). On the other hand, for malts containing more β-amylase (89M) (Fig. 1a), no such significant changes were observed. During mashing, initially α-amylase randomly hydrolyzes internal (1 → 4)-α glycosidic bonds to produce an array of linear and branched dextrins, which will be further hydrolyzed by α-amylase. Then β-amylase sequentially removes units of maltose from the non-reducing ends of those large dextrins, while limit dextrinase could rapidly hydrolyze the (1 → 6)-α branch points in these limit dextrins to produce linear dextrins which are then available for β-amylase to degrade further to maltose (MacGregor et al., 1999). For malt containing more β-amylase (89M), this enzyme is already in excess. Consequently, even though more β-amylase and limit dextrinase were released after Neutrase addition, this may not necessarily mean more sugars would be released. On contrary, increased enzyme activity of β-amylase could have, to some extent, inhibited the hydrolysis of the larger starch molecules during mashing, as reported by Ao et al. (2007). This hypothesis is supported by the existence of more larger starch molecules remaining undegraded in BSG after mashing, as shown in Fig. 4. This is consistent with the findings of MacGregor et al. (1999), who reported that it would be more beneficial to improve the heat stability of β-amylase already present rather than to select for barleys with higher β-amylase levels, if one were only attempting to increase levels of fermentable sugars in the wort.

For BSG produced without the addition of Neutrase, Bi et al. (2018) used proteomics to investigate the remaining undegraded protein compositions after mashing. They found that, except for the presence of a high amount of β - amylase, both hordein (especially B- hordeins and C- hordeins) and globulins were the most abundant proteins in BSG. It was found that although was not as effective as protease S and protease K, Neutrase could still efficiently hydrolyze malt hordeins (especially B-hordeins) and partially hydrolyze globulins. Further, in a previous study, we found that the mixture of hordeins and globulins in barley could significantly inhibit starch *in vitro* degradation through binding interactions between protein and α-amylase (Yu, Zou, et al., 2018). Combining these findings, we propose that hordein and/globulin hydrolysis through Neutrase should be the main reason for the effects of malt protein removal on starch molecular structural change during mashing. More experiments, such as using SDS-PAGE, could test this hypothesis.

5. Conclusions

This research has examined the changes caused by the removal of malt protein on mashing performance, considering both wort fermentable sugar content and the molecular fine structures of wort-soluble starches. Our results found that Neutrase addition could significantly reduce the amount, and also the molecular sizes and molecular chain lengths, of wort-soluble starches. However, the effects of protein removal on the production of fermentable sugars, including maltose and

maltotriose, largely depend on the original enzyme activity of the malt.

Protein content is currently a major criterion for the choice of a particular barley variety for beer production. However, it is likely that the effects of malt protein on mashing efficiency, particularly β-amylase, is also important in altering starch degradation during mashing. That is, for malt containing lower β-amylase enzyme activity, protein removal could not only significantly increase wort fermentable sugar, but could significantly decrease the content of soluble starches in wort, particularly of those with smaller molecular sizes and short chains ($R_h < 3.5$ nm, DP < 30, Fig. 3). On the other hand, for malts containing more β-amylase, the removal of malt protein would not significantly increase wort-fermentable sugar content, as β-amylase would already be in excess, but can still reduce both the content and the molecular sizes of soluble starches remaining in the wort (Fig. 1, Table 2).

Overall, this study reveals the fact that protein content is not the sole determining factor controlling mashing performance. Combined with previous results (G Fox, Yu, Nischwitz, & Harasymow, 2019; Yu et al., 2019), the role of malt starch structure should be taken into consideration when evaluating the brewing quality of a barley variety.

Acknowledgements

We gratefully acknowledge the support of the 2017 Jiangsu Innovation and Entrepreneurship Talents Program and of a National Science Foundation of China grant C1304013151101138.

Wenwen Yu gratefully acknowledges the support of the China Scholarship Council, the University of Queensland and Jinan University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2019.105423.

References

Ao, Z., Simsek, S., Zhang, G., Venkatachalam, M., Reuhs, B. L., & Hamaker, B. R. (2007). Starch with a slow digestion property produced by altering its chain length, branch density, and crystalline structure. *Journal of Agricultural and Food Chemistry, 55*(11), 4540–4547.

Asare, E. K., Jaiswal, S., Maley, J., Baga, M., Sammynaiken, R., Rossnagel, B. G., et al. (2011). Barley grain constituents, starch composition, and structure affect starch in vitro enzymatic hydrolysis. *Journal of Agricultural and Food Chemistry, 59*(9), 4743–4754.

Bi, X., Ye, L., Lau, A., Kok, Y. J., Zheng, L., Ng, D., et al. (2018). Proteomic profiling of barley spent grains guides enzymatic solubilization of the remaining proteins. *Applied Microbiology and Biotechnology, 102*(9), 4159–4170.

Bøjstrup, M., Marri, L., Lok, F., & Hindsgaul, O. (2015). A chromogenic assay suitable for high-throughput determination of limit dextrinase activity in barley malt extracts. *Journal of Agricultural and Food Chemistry, 63*(50), 10873–10878.

Castro, J. V., Ward, R. M., Gilbert, R. G., & Fitzgerald, M. A. (2005). Measurement of the molecular weight distribution of debranched starch. *Biomacromolecules, 6*(4), 2260–2270.

Chandrashekar, A., & Kirleis, A. (1988). Influence of protein on starch gelatinization in sorghum. *Cereal Chemistry, 65*(6), 457–462.

Fox, G. (2016). Infrared spectral analysis of sugar profiles of worts from varying grist to liquor ratios using infusion and ramping mash styles. *Journal of the Institute of Brewing, 122*(3), 437–445.

Fox, G., Yu, W., Nischwitz, R., & Harasymow, S. (2019). Variation in maltose in sweet wort from barley malt and rice adjuncts with differences in amylose structure. *Journal of the Institute of Brewing, 125*(1), 18–27.

Gong, B., Cheng, L., Gilbert, R. G., & Li, C. (2019). Distribution of short to medium amylose chains are major controllers of in vitro digestion of retrograded rice starch. *Food Hydrocolloids, 96*, 634–643.

Guo, L. (2018). In vitro amylase hydrolysis of amylopectins from cereal starches based on molecular structure of amylopectins. *Food Hydrocolloids, 77*, 238–247.

Hu, S., Dong, J., Fan, W., Yu, J., Yin, H., Huang, S., et al. (2014). The influence of proteolytic and cytolytic enzymes on starch degradation during mashing. *Journal of the Institute of Brewing, 120*(4), 379–384.

Hu, S., Yu, J., Dong, J., Evans, D. E., Liu, J., Huang, S., et al. (2014). Relationship between levels of diastatic power enzymes and wort sugar production from different barley cultivars during the commercial mashing process of brewing. *Starch Staerke, 66*(7–8), 615–623.

Kammoun, R., Bejar, S., & Ellouz, R. (2003). Protein size distribution and inhibitory effect of wheat hydrolysates on Neutrase®. *Bioresource Technology, 90*(3), 249–254.

Lund, M. N., Petersen, M. A., Andersen, M. L., & Lunde, C. (2015). Effect of protease treatment during mashing on protein-derived thiol content and flavor stability of beer during storage. *Journal of the American Society of Brewing Chemists, 73*(3), 287–295.

MacGregor, A., Bazin, S., Macri, L., & Babb, J. (1999). Modelling the contribution ofAlpha-amylase, beta-amylase and limit dextrinase to starch degradation during mashing. *Journal of Cereal Science, 29*(2), 161–169.

Pliansrithong, P., Usansa, U., & Wanapu, C. (2013). Increasing of Nitrogenous substances in wort by using commercial enzymes and modifying mashing method. *International Journal of Bioscience, Biochemistry and Bioinformatics, 3*(4), 404–407.

Schulz, B. L., Phung, T. K., Bruschi, M., Janusz, A., Stewart, J., Meehan, J., et al. (2018). Process proteomics of beer reveals a dynamic proteome with extensive modifications. *Journal of Proteome Research, 17*(4), 1647–1653.

Serna-saldívar, S., Espinosa-Ramírez, J., & Esther, P. (2014). Maltose and glucose utilization during fermentation of barley and sorghum lager beers as affected by b-amylase or amyloglucosidase addition. *Journal of Cereal Science, 60*, 602–609.

Slack, P., Baxter, E., & Wainwright, T. (1979). Inhibition by hordein of starch degradation. *Journal of Institue of Brewing, 85*(2), 112–114.

Syahariza, Z. A., Sar, S., Tizzotti, M., Hasjim, J., & Gilbert, R. G. (2013). The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains. *Food Chemistry, 136*(2), 742–749.

Tao, K., Li, C., Yu, W., Gilbert, R. G., & Li, E. (2019). How amylose molecular fine structure of rice starch affects functional properties. *Carbohydrate Polymers, 204*, 24–31.

Vilaplana, F., & Gilbert, R. G. (2010). Characterization of branched polysaccharides using multiple-detection size separation techniques. *Journal of Separation Science, 33*(22), 3537–3554.

Wang, K., Hasjim, J., Wu, A. C., Henry, R. J., & Gilbert, R. G. (2014). Variation in amylose fine structure of starches from different botanical sources. *Journal of Agricultural and Food Chemistry, 62*(19), 4443–4453.

Wang, J., Zhang, G., Chen, J., & Wu, F. (2004). The changes of β-glucan content and β-glucanase activity in barley before and after malting and their relationships to malt qualities. *Food Chemistry, 86*(2), 223–228.

Yu, W., Quek, W., Li, C., Gilbert, R., & Fox, G. (2018). Effects of the starch molecular structures in barley malts and rice adjuncts on brewing performance. *Fermentatio, 4*(4), 103.

Yu, W., Tao, K., Gidley, M. J., Fox, G. P., & Gilbert, R. G. (2019). Molecular brewing: Molecular structural effects involved in barley malting and mashing. *Carbohydrate Polymers, 206*, 583–592.

Yu, W., Zou, W., Dhital, S., Wu, P., Gidley, M. J., Fox, G. P., et al. (2018). The adsorption of α-amylase on barley proteins affects the in vitro digestion of starch in barley flour. *Food Chemistry, 241*, 493–501.

Zou, W., Sissons, M., Gidley, M. J., Gilbert, R. G., & Warren, F. J. (2015). Combined techniques for characterising pasta structure reveals how the gluten network slows enzymic digestion rate. *Food Chemistry, 188*, 559–568.

Zou, W., Sissons, M., Warren, F. J., Gidley, M. J., & Gilbert, R. G. (2016). Compact structure and proteins of pasta retard in vitro digestive evolution of branched starch molecular structure. *Carbohydrate Polymers, 152*, 441–449.